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(54) Title: TARGETING VIRUSES AND CELLS FOR SELECTIVE INTERNALIZATION BY CELLS

(57) Abstract

Viruses or cells are targeted for selective internalization into a target *in vivo*. A molecule specific for a receptor on the surface of the target cell is introduced onto the surface of the virus or cell. The modified virus or cell binds the receptor *in vivo* and is internalized by the target cell. The method provides vectors for selective delivery of nucleic acids to specific cell types *in vivo* and a means to alter the tropism of an infectious agent.

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TARGETING VIRUSES AND CELLS FOR SELECTIVE INTERNALIZATION BY CELLS

Background of the Invention

Viruses represent a natural and efficient means for the introduction of foreign genes into cells. For this reason, they are useful tools for the study of genes, and gene regulation *in vitro* and for gene therapy. However, most viruses have broad cell specificity and can infect a wide variety of cell types. This can lead to foreign gene expression in many tissues, some of which may be undesirable, especially for clinical applications.

Generally, viral infection is mediated by interactions between viral envelopes and plasma membranes of target cells. In many cases, specific viral structures are recognized and bound by cellular receptors. For example, HIV employs envelope glycoproteins to bind to helper T lymphocytes via CD4 (T4) receptors. Dalgleich, A.G., *et al.* *Nature* 312:763-767 (1984). These interactions have been shown to be responsible for the observed species and organ specificity.

Some investigators have shown that virus specificity can be redirected by attaching antibodies to viruses. For example, Goud, B., et al. Virology 163:251-254 (1988) linked anti-transferrin receptor antibodies to obtain delivery of a retrovirus to human cells bearing the transferrin receptor. However, while binding and internalization occurred, infection and replication did not. A means for targeting viral or other types of nucleic acid vectors containing foreign genes to a target cell and obtaining infection and replication of the virus would be useful in gene therapy.

Summary of the Invention

The invention pertains to a method of targeting a virus or a cell to a target cell for selective internalization in vivo (or in vitro) by the cell and to modified viruses and cells which are targeted for selective internalization by a target cell.

A virus or cell is targeted to the target cell for internalization by introducing a receptor-specific molecule onto the surface of the virus or cell to produce a modified virus or cell which specifically binds to a receptor on the surface of the target cell. The modified virus or cell can be administered to an organism where it binds selectively to the receptor of the target cell. The receptor-binding results in internalization by the target cell.

The cellular receptor can be a receptor which mediates endocytosis of a bound ligand such as the asialoglycoprotein receptor of hepatocytes and the receptor-specific molecule can be a natural or synthetic ligand for the receptor. The receptor-specific molecule can be introduced onto the surface of the virus or cell (e.g., onto a viral envelope or cellular membrane) by chemically coupling it, either directly or through bridging agents, to the surface or by treating the surface to expose the molecule for receptor recognition.

The method of this invention can be used to produce viral or cellular vectors for selective delivery of material such as nucleic acid (genes) to a target cell. For example, exogenous genes can be incorporated and expressed selectively in a target cell. These vectors can be used in gene therapy and in other applications which call for selective genetic alteration of cells.

The method also provides a means for altering the natural tropism of an infective agent such as a virus or bacterium. An infective agent can be modified so that it will infect a cell which, in unmodified form, it would not normally infect. In this way, animal models of human diseases which do not have adequate experimental animal counterparts can be developed for study of the diseases. For example, an ecotropic human pathogen (such as the hepatitis or AIDS virus) can be modified to infect a non-human host to produce an experimental system for study of the pathogen and the disease.

Brief Description of the Figures

Figure 1 shows in situ ß-galactosidase expression in NIH 3T3, HepG2 and SK Hepl cells treated separately with unmodified or modified murine leukemia virus.

Figure 2 shows internalization of ^{35}S-biolabeled modified Moloney murine leukemia virus.

Figure 3 shows a chromatogram of asialooro-mucoid-complexed Psi2 virus on Sephadex G150.

Figure 4 shows the ß-galactosidase activity of various cells exposed to Psi2 virus-asialoglyco-protein conjugate.

Detailed Description of the Invention

A virus or cell is targeted for selective internalization into a target cell by modifying the surface of the virus or cell to introduce a molecule which specifically binds to a surface receptor of the target cell. The cellular surface receptor is one which will mediate internalization of the targeted virus or cell. The modified virus or cell binds to the receptor of the target cell in vivo and is internalized by the cell.

According to the method of this invention, viruses can be modified to infect specific target cells. Such modified viruses can be used to selectively deliver exogenous, functional DNA to a target cell in order confer a new biological or biochemical property upon the cell or to abrogate an existing property. In addition, the tropism of a virus can be altered or redirected to target infectivity to a cell type or types not normally infected by the virus in natural (or unaltered) form.

A variety of different enveloped viruses can be targeted by the method of this invention. The viruses can be RNA (retroviruses) or DNA viruses (e.g., hepatitis virus, adenovirus). The virus can be replication defective or otherwise defective in structure or function. For example, viral particles either essentially or completely devoid of genomic nucleic acid (e.g., "empty" viral envelope) can also be targeted.

The present method also provides a means of targeting cells. These include cellular organisms such as bacteria, protozoa or trypanosomes whose tropism can be altered. In addition, mammalian cells can be targeted.

The receptor-specific molecule can be a ligand for the surface receptor of the target cell. Preferably, the molecule is a ligand for a cellular surface receptor which mediates internalization of the ligand by the process of endocytosis, such as the asialoglycoprotein receptor of hepatocytes.

Glycoproteins having certain exposed terminal carbohydrate groups can be used as receptor-specific molecules. For specific targeting to hepatocytes, asialoglycoprotein (galactose-terminal) ligands are preferred. Examples of asialoglycoproteins include asialoorosomucoid or asialofetuin. Other useful galactose-terminal carbohydrates for hepatocyte targeting include carbohydrate trees obtained from natural glycoproteins, especially tri- and tetra-antennary structures that either contain terminal galactose residues or can be enzymatically treated to expose terminal galactose residues. In addition, naturally occurring plant carbohydrates such as arabinogalactan can be used.

For targeting other receptors, other types of carbohydrates can be used. For example, mannose and mannose-6 phosphate or carbohydrates having these terminal carbohydrate structures could used to target macrophages or endothelial cells.

Other receptor ligands such as peptide hormones could also be used to target viruses or cells to corresponding receptors. These include insulin, glucagon, gastrin polypeptides and their respective receptors.

Alternatively, the receptor-specific molecule can be a receptor or receptor-like molecule, such as an antibody, which binds a ligand (e.g., antigen) on the cell surface. Antibodies specific for cellular surface receptors can be produced by standard procedures.

The receptor-specific molecule is introduced onto the surface of the virus or cell so that it will be recognized by the cognate cellular surface receptor. For example, the receptor-specific molecule can be introduced onto the envelope of a virus or the membrane of a cell. In general, the molecule will be coupled to (or exposed on) a proteinaceous component of the surface but other components may be used.

The receptor-specific molecule can be introduced onto the surface of the virus or cell by different means. Preferably, the receptor-specific molecule is chemically coupled to the surface. For example, galactose moieties (ligand for the asialoglycoprotein receptor) can be covalently coupled to viral or cellular surface proteins by lactosamination, reductive amination, or via

iminomethoxyethyl derivatives. In other embodiments, the receptor-specific molecule can be chemically coupled to components of the surface of the virus or cell through bridging agents such as biotin and avidin. For instance, a biotinylated receptor-specific molecule can be linked through avidin or streptavidin to a biotinylated surface component of the virus or cell.

Alternatively, the virus or cell can be chemically treated to expose a receptor-specific molecule on the surface. Surface polycarbohydrates can be enzymatically cleaved to expose desired carbohydrate residues (e.g., galactose residues) as terminal residues for specific receptor recognition and binding. For example, neurominidase treatment of certain polycarbohydrates leaves exposed terminal galactose residues in a tri- or tetra-antennary arrangement.

The modified virus or cell is administered *in vivo*, generally in an amount sufficient to saturate receptors of the target cell and thereby maximize uptake by the cell. They can be administered parenterally (typically intravenously) in a physiologically acceptable vehicle such as normal saline.

The method of this invention can be used to selectively deliver nucleic acid (DNA or RNA) to a target cell *in vivo* (or *in vitro*) so that it is expressed in the cell. The nucleic acid can be an exogenous gene, a genetic regulatory element or an antisense inhibitor of gene function. The nucleic acid is incorporated into a viral vector which has been modified, according to the method of this

invention, to target it to the cell. Preferred viral vectors for delivery of foreign genes in vivo (or ex vivo) are retroviruses. The targeted viral vector is administered in vivo, as described, where it is selectively taken up by the target cell.

The method of this invention can be used to alter the natural tropism of an infectious agent. Ecotropic (species-restricted) agents can be made to infect species which they normally, in unmodified form, do not infect. The ability to target the infectivity of an infectious agent can be used to develop new experimental systems for the study of human infectious diseases to produce cells that can correct genetic defects in vivo, or target a corrective gene in vivo.

Certain pathogenic viruses such as hepatitis virus or human immunodeficiency virus infect only human cells. By the method of this invention, such viruses can be modified to enable them to infect experimental animals such as rodents. For example, the hepatitis virus which infects only human liver cells, can be modified so that it will infect non-human liver cells. To develop rodent models of hepatitis, for example, a ligand for rodent asialoglycoprotein receptor (e.g., galactose) can be introduced onto the surface of the hepatitis virus. This yields a modified hepatitis virus which will infect rodent liver cells. This modified hepatitis virus which can infect a rodent and the infected rodent or rodent cells, provides an experimental animal system for study of the hepatitis virus.

The invention is illustrated further by the following examples.

EXAMPLE 1

Chemical Modification and Alteration of Host Cell Specificity of a Retrovirus

A model retroviral system was used. The virus, an ecotropic, replication-defective, Moloney murine leukemia virus containing the gene for bacterial ß-galactosidase produced in a ψ cre cell line was kindly provided by Dr. James Wilson, University of Michigan. Wilson, J.M., et al. Proc. Natl. Acad. Sci. USA 87:439-443 (1990). Under normal circumstances, this virus infects only rodent cells. Wilson, J.M., et al. Proc. Natl. Acad. Sci. USA 85:3014-3018 (1988); Goud, B., et al. Virology 163:251-254 (1988). The producer cells were grown in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated calf serum (GIBCO). To prepare virus with as little contamination as possible from serum proteins, producer cells were cultured in serum-free Dulbecco's modified Eagle's medium for 3 days. Using this viral preparation, two strategies were developed for the modification of the surface of the harvested virus: A) chemical coupling of galactose residues to the virus and B) chemical coupling of an asialoglycoprotein to the virus.

A. LACTOSAMINATION OF RETROVIRUSES

Virus was isolated from the culture medium according to the method of Goud, B., et al. Virology 163:251-254 (1988), but modified to permit coupling of lactose during the isolation procedure. In brief, virus-containing medium was applied on a 10-20% sugar gradient in which α-lactose was substituted for sucrose (Sigma, St. Louis, MO) in 10 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, and was ultracentrifuged (LB-55, Beckman Instruments, San Ramon, CA) at 40,000 rpm in VTi 55 rotor (Beckman) at 4°C for 17 hours. Samples were adjusted to various pHs, 7.4-8.4, prior to centrifugation in order to determine optimal conditions for modification. After centrifugation, the bottom fraction of the lactose gradient containing 3.0 mg of protein (0.1 mg viral RNA) was reacted with sodium cyanoborohydride (Sigma) as described previously. Goud, B., et al. Virology 163:251-254 (1988). Following dialysis against minimum essential medium at 4°C for 24 hours, the samples were sterilized by passage through 0.45μ filters (Gelman Science Co., Ann Arbor, MI). Quantitation of the amount of virus present in samples prior to exposure to cells was determined by protein assay (Bio-Rad, Los Angeles, CA) according to the manufacturer's instructions, and confirmed by RNA assay (Nunro, H.N. and Fleck, A. Meth. Biochem. Anal. 14:113-176 (1966)) after RNA extraction. Chomczynski, P. and Sacci, N., Anal. Biochem. 162:156-159 (1987). Fetal bovine serum (GIBCO) was added subsequently to make a 10% solution. Except for stability experiments, all samples were used

immediately after preparation. Viability of unmodified virus preparations was determined by transfection assays in NIH 3T3 mouse fibroblasts using limiting dilutions of viral stock (Danos, O. and Mulligan, R.C. Proc. Natl. Acad. Sci. USA 85:6460-6464 (1988)) and quantitated by determination of positive cells stained with X-gal. Sanes, J.R., et al. EMBO J. 5:3133-3142 (1986).

For uptake studies, virus was biosynthetically labeled by incubation of producer cells (5.0×10^6 cells) in 50% serum-free and 50% serum- and methionine-free Dulbecco's modified Eagle's medium containing 10 µCi/ml ^{35}S-methionine (Amersham, Arlington Heights, IL) for 3 days. Virus was isolated from supernatants and modified as described above followed by dialysis against minimum essential medium.

Cells and Cell Culture

To evaluate the effects of chemical modification on viral infection specificity, several cell lines were employed: human hepatoma cell lines, HepG2, asialoglycoprotein receptor (+) (Schwartz, A.L., et al. J. Biol. Chem. 256:8878-8881 (1981)) obtained from B.B. Knowles, Wistar Institute, Philadelphia, PA; and SK Hepl, receptor (-) from D.A. Shafritz, Albert Einstein College, of Medicine, Bronx, NY; a rat hepatoma cell line, Morris 7777, receptor (-) (Wu. G.Y., et al. J. Biol. Chem. 263: 4719-4723 (1988)); and a murine fibroblast cell line NIH 3T3 (Goud, B., et al. Virology 163:251-254 (1988)) which is also asialoglycoprotein receptor (-). The latter two cell lines were purchased from

American Type Culture Collection (Rockville, MD). All were maintained in Eagle's minimum essential medium supplemented with 10% heat inactivated fetal bovine serum at 37°C under 5% CO_2.

Assays for Viral Infection and Functional Gene Expression

In order to determine whether virus remained infectious and functional after chemical modification, the two human and two rodent cell lines were exposed to modified and unmodified virus followed by measurement of cellular ß-galactosidase activity. Target cells were plated at a density of 0.5-2.0 x 10^5 cells/ml in 60 mm plastic dishes (Falcon Scientific Co, Lincoln Park, NJ). Equal amounts, 16.7 µg RNA, (0.5 mg viral protein) of modified and unmodified virus, in Dulbecco's modified Eagle's medium were added to the culture medium and exposed to cells for 5 days at 37°C under 5% CO_2. Cells were assayed for ß-galactosidase activity as a measure of foreign gene expression according to the method of Gorman (Gorman, C. DNA Cloning, vol. 2 eds, Glover, D.M. IRL Press, Washington D.C. pp 157-158 (1986)). In brief, cell monolayers (approximately 1x10^6 cells/60 mm dish) were washed with phosphate buffered saline, then lysed. The lysate, 0.1 ml, was reacted with o-nitrophenyl-galactopyranoside (ONPG, Sigma) and ß-galactosidase activity quantitated by absorbance at 420 nm after addition of Na_2CO_3 to terminate the reaction. Results were expressed in U/mg of cellular protein according to the method by Norton,

P.A. and Coffin, J.M. Mol. Cell. Biol. 5:281-290 (1985), using purified E. coli ß-galactosidase (Sigma) activity as a standard. Protein concentrations of the cellular samples were determined using a Bio-Rad Protein Assay Kit (Bio-Rad) following the manufacturer's instructions. For competition experiments, virus was added to the cell media together with a 100-fold molar excess of a natural asialoglycoprotein, asialoorosomucoid, prepared by desialylation (Oka, J.A., and Weigel, P.H. J. Biol. Chem. 258: 10253-10262 (1983)) of orosomucoid as previously described by Whitehead., D.H., and Sammons, H.G. Biochim. Biophys. Acta 124:209-211 (1966). Background enzyme activity was determined in corresponding untreated cells and subtracted from the values of viral-treated samples. All assays were performed in triplicate and the results expressed as means ± S.E.

Table 1 shows that unmodified virus did not produce enzymatic activity in human HepG2 or SK Hepl cells as expected from the ecotropism of the virus. Also, modified virus did not produce ß-galactosidase activity in SK Hepl, asialoglycoprotein receptor (-) cells. However, modified virus did produce high ß-galactosidase activity, 71.2 ± 4.8U/mg of cellular protein, in human HepG2, asialoglycoprotein receptor (+) cells. Furthermore, this enzymatic activity was completely suppressed by addition of a large molar excess of asialoorosomucoid, supporting the notion that the transfection by modified virus was, in fact, mediated by asialoglycoprotein receptors. As expected from the ecotropism, ß-galactosidase

activity was high, 50.6 ± 5.2. in Morris 7777 rat cells after exposure to unmodified virus. Interestingly, ß-galactosidase activity in these same cells was significantly lower when exposed to the same amount of modified virus. The same tendency was seen in originally susceptible murine NIH 3T3 cell as enzymatic activity after exposure to unmodified virus, 56.7 ± 1.8, was more than double that following exposure to modified virus 27.0 ± 0.9.

The coupling reaction linking lactose to protein has been shown to be enhanced under alkaline conditions. Schwartz, B.A. and Gray, G.R. Arch. Biochem. Biophys. 181:542-549 (1977). However, such conditions could be detrimental to the virus. To determine the optimal pH that results in modified, yet functional vectors, virus modified at different pHs were administered to HepG2 cells, and ß-galactosidase activity measured. Table 2 shows that enzymatic activity rose from 50.3 ± 1.2, for virus modified at pH 7.4; to 71.2 ± 4.8, for virus modified at pH 8.0. However, activity was significantly lower, 25.1 ± 2.4, in cells treated with virus modified at pH 8.4.

Table 1

Cellular ß-Galactosidase Activity
Following Exposure to Viral Preparations+

ß-Galactosidase Activity*

Mean ± S.E. (U/mg)

Cell Line (Source)	AsG Receptor Status	Unmodified Virus	Modified Virus	Modified Virus + ASOR**
HepG2 (human)	(+)	1.8 ± 1.9	71.2 ± 4.8	2.9 ± 1.1
SK Hep1 (human)	(-)	1.7 ± 3.4	0.8 ± 4.6	1.6 ± 2.5
Morris 7777 (rat)	(-)	50.6 ± 5.2	16.3 ± 4.4	15.7 ± 4.7
NIH 3T3 (mouse)	(-)	52.1 ± 4.9	15.4 ± 1.1	16.3 ± 3.9

+ virus was modified at pH 8.0 then incubated with cells for 5 days.

* calculated as the difference in activity between treated and untreated cells.

** asialoorosomucoid (ASOR) in 100-fold molar excess.

AsG, Asialoglycoprotein

Table 2

Effect of the pH During Modification of Viral Transfection in HepG2 Cells

Specific ß-Galactosidase Activity
(Mean ± S.E. U/mg protein)*

pH	Modified Virus	Modified Virus + Asialoorosomucoid**
7.0	50.3 ± 1.2	6.4 ± 1.9
8.0	71.8 ± 4.1	4.9 ± 0.4
8.4	25.1 ± 2.4	0.0 ± 1.6

* after 5 days of exposure to modified virus.

** ß-galactosidase activity of samples treated with modified virus plus a 100-fold molar excess of asialoorosomucoid.

Histochemical Staining to Demonstrate ß-Galactosidase Activity

To confirm the colorimetric results, and to determine the fraction of cells that expressed the ß-galactosidase gene after exposure to viral samples, histochemical staining of *in situ* ß-galactosidase activity was performed according to the method of Sanes *et al.* EMBO J. 5:3133-3142 (1986). In brief, cultured cells in 35 mm dishes containing 0.5 -1 x 10^6 cells treated for 5 days with equal amounts, 8.4 µg of viral RNA (0.3 mg viral protein), of modified or unmodified virus. Cells were fixed in 0.5% glutaraldehyde (Sigma), phosphate buffered saline, then incubated with 1 mM $MgCl_2$, phosphate buffered saline, and overlaid with 1mg/ml 4-Cl-5-Br-3-indoylyl-ß-galactosidase (X-gal) (BRL, Washington, D.C.), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$ in phosphate buffered saline. After incubation at 37°C for 1 hour, the dishes were washed in phosphate buffered saline to quench the reaction and evaluated by counting positive (blue) cells under a light microscope and the results expressed as the percent of positive/10 high power fields.

In situ staining for ß-galactosidase activity in cells treated with various viral preparations is shown in Figure 1. In rodent NIH 3T3 cells treated with unmodified virus, panel B, 12.6% were positive for ß-galactosidase activity using the X-gal stain. Background staining in untreated NIH 3T3 cells was not detectable, panel A. After exposure to modified virus, only 3.6% were positive under otherwise

identical conditions, panel C. Human SK Hepl cells that were untreated, panel H, or exposed to either unmodified virus, panel I, or modified virus, panel J, failed to develop detectable staining. Similarly, HepG2, asialoglycoprotein receptor (+) cells treated with unmodified virus, panel E, did not develop evidence of significant ß-galactosidase activity. However, HepG2, receptor (+) cells treated with modified virus, panel F, did develop substantial staining. Microscopic counting revealed that 36.4 % of the HepG2, cells possessed detectable marker enzyme. The observed color development was completely suppressed by addition of a 100-fold molar excess asialoorosomucoid to compete for uptake by asialoglycoprotein receptors, panel G, indicating involvement of asialoglycoprotein receptors in the transfection process.

Assays for Cellular Uptake of Virus

To determine whether the modified virus was actually taken up by cells and, if so, whether asialoglycoprotein receptors were involved, HepG2, SK Hepl and Morris 7777 cells, $5.0x10^5$ cells/35 mm dish, were incubated at 37°C in serum-free Dulbecco's modified Eagle's medium containing ^{35}S-biolabeled, modified virus, 3.3 µg viral RNA (98 µg viral protein) (Watanabe, N., et al. Cancer Immunol. Immunother. 28:157-163 (1989)) with a specific activity of $6.1x10^5$ cpm/mg viral RNA. At various times, medium was removed, and cells were chilled to 4°C, washed with ice-cold minimum essential medium containing 1mg/ml bovine serum albumin. Surface-bound radioactivity was stripped

with cold 0.5 ml phosphate buffered saline, pH 7.2 containing 0.4% trypsin, 0.02% EDTA and separated from cells by centrifugation. The cell pellet was solubilized in 0.2 N NaOH and Poly-Fluor (Packard, Chicago, IL), and trypsin-EDTA resistant (internalized) radioactivity was measured by scintillation counting (TRI-CARB 4530, Packard). Schwartz, A.L., et al. J. Biol. Chem. 256:8878-8881 (1981). Non-specific uptake was measured in the presence of a 100-fold molar excess of asialoorosomucoid, and specific uptake calculated as the difference between total and non-specific measurements. All assays were performed in triplicate and the results expressed as means ± S.E. in terms of ng viral RNA/10^5 cells as a function of time.

Figure 2 shows that, of the two human and one rodent cell lines, only the human HepG2 asialoglycoprotein receptor (+) cells demonstrated significant specific uptake of labeled virus. Counts resistant to EDTA and trypsin, increased as a function of time and continued to rise linearly through 120 minutes of incubation at a rate of approximately 800 ng viral protein/hr/10^5 cells. These data further support the notion that the observed expression of the galactosidase gene by modified virus was in fact due to internalization of the virus by asialoglycoprotein receptors.

Stability of Modified Virus

To assess the stability of modified virus, samples of freshly prepared sterile, modified virus were incubated in serum-free Dulbecco's modified

Eagle's medium at 4°C and 25°C. At various times, samples were added to the medium of HepG2 cells and incubated for 5 days. Cells were then assayed for ß-galactosidase activity by colorimetric assay as described above. All assays were performed in triplicate and the results expressed as means ± S.E. in terms of U/mg cell protein normalized for the amount of virus added as a function of time of incubation. Table 3 shows that enzymatic activity at both 4°C and 25°C, decreased with time to approximately 50% of original activity after 48 hours.

Table 3

Stability of Modified Virus

Temperature	Time of Incubation	Specific Activity (Mean + S.E. U/mg protein)*
4°C	0 hr	50.3 ± 1.2
	24 hr	42.0 ± 5.6
	48 hr	22.6 ± 2.5
25°C	24 hr	37.1 ± 2.8
	48 hr	17.6 ± 1.1

* after 5 days of exposure to modified virus

Specific ß-galactosidase activity was calculated as the difference between samples treated with virus alone, and samples treated with modified virus plus a 100-fold molar excess of asialoorosomucoid.

The coupling of lactose to proteins to target artificial asialoglycoproteins is based on the specificity of sodium cyanoborohydride to reduce Schiff's bases formed between aldehyde and amino groups to render the bonds irreversible. Treatment of viruses with aldehydes is not always similarly benign. For example formaldehyde has been used to inactivate viruses in the production of vaccines. Buynak, E.B., et al. J. Am. Med. Assoc. 235: 2832-2834 (1976). The data presented here indicate that under the conditions described, the modification process results not only in altered specificity of infection, but also results in preservation of viral gene expression. Furthermore, the data indicate that the production of modified yet functional virus increased with increasing pH of the modification reaction up to a limit of approximately 8.0, beyond which the function of the virus became compromised.

Many retroviruses have been shown to enter cells normally via endocytosis and are thought to introduce their genetic material during an acidification step in the pathway. Andersen, K.B. and Nexo, P.A. Virology 125:85-98 (1983). Although the asialoglycoprotein endocytotic pathway is ultimately degradative with delivery of ligands to lysosomes (Tolleshaug, H., et al. Biochim. Biophys. Acta 585:71-84 (1979)), early in the internalization process, endosomal endocytotic compartments are acidified prior to fusion with lysosomes. Tycko, B. and Maxfield, R.F. Cell 28:643-651 (1982). This period of acid exposure may be analogous to the

natural route of entry for some viruses (Nussbaum, O., and Loyter, A. FEBS Lett. 221:61-67 (1987)) and may provide the requisite conditions for acid-mediated fusion of the viral envelope of endosomal membrane prior to destruction of the virus. Helenius, A. Biol. Cell 51:181-186 (1984). The fact that modified virus is still able to introduce its genome into target cells suggests that the process of chemical modification did not abolish the function of those elements of the virus.

B. VIRUS-ASIALOGLYCOPROTEIN CONJUGATES

Crude preparations of virus obtained by low-speed centrifugation of medium from producer BAG cells followed by high-speed centrifugation through a discontinuous sucrose gradient as described previously was dialyzed against 0.9% saline, pH 7.5, at 4°C. After dialysis, NHS-LC-biotin (Pierce Chemical Co., Rockville, IL) was reacted with the virus (0.1 mg/ml of virus) at room temperature for four hours. The sample was then dialyzed against 0.9% saline, pH 7.5 at 4°C. Asialoorosomucoid (AsOR) was obtained by desialylation of serum orosomucoid originally derived from pooled human serum. Whitehead, D.H. and Sammons, H.P. Biochem. Biophys. Acta 124:209 (1966). AsOR 0.1 mg was added to 1.0 mg of virus, thoroughly mixed and then 1.0 mg of avidin per mg of virus was added and allowed to incubate at room temperature for four hours. The complex was then dialyzed against Modified Eagle's Medium. Complexed virus was purified on a Sephadex G150 molecular sieve column. To determine conditions for purification, a viral complex was

prepared in which asialoorosomucoid was labeled with ^{125}I. Figure 3 shows that asialoorosomucoid alone was eluted from the column beginning at fraction number 32. Avidin, as detected by its optical density at 280 nm, eluted slightly later beginning at tube 33. However, unlabeled virus alone was much larger than either of the other two proteins and was eluted earlier with a peak at tube 29. The column was able to completely resolve virus from asialoorosomucoid and avidin. Figure 3 also shows that this virus complexed with ^{125}I-labeled AsOR mediated by biotin-avidin bonds, the radioactivity from the AsOR moved to the same position as expected for the intact virus, namely with a peak at tube 29. These data indicate that some labeled asialoorosomucoid was bound by the virus and migrated with it through the column.

In order to determine whether this complex could be used to target gene expression specifically to asialoglycoprotein receptor (+) cells, conjugated virus was incubated for 10 days with each of five cell lines: Hep G2, receptor (+); Huh-7, receptor (+); SK Hepl, human hepatoma receptor (-); Mahlavu, receptor (-) and Morris 7777, rat hepatoma receptor (-) cells. Figure 4, lane 1 shows that Hep G2 receptor (+) cells treated with conjugate had beta-galactosidase activity at a level of 2.3 units/mg of cell protein which is approximately 50% of the activity of the producer cell line, BAG shown in lane 11. Hep G2 cells without treatment were at a level of 1.81 units/mg. Huh-7 receptor (+) cells treated with conjugate had higher levels of beta-galactosidase, 3.8 units/mg as shown in lane 3

compared to those cells treated with biotinylated virus without asialoorosomucoid present in a complex shown in lane 4. This was similar to the levels obtained from these cells that were not treated at all as seen in lane 5. Lane 6 shows that Mahlavu receptor (-) cells treated with conjugate did not have any significant beta-galactosidase activity compared to those same cells that were untreated shown in lane 7. Similarly lanes 8 and 9 show that Morris 7777 cells treated with other conjugate or biotinylated virus without asialoorosomucoid, lanes 8 and 9 respectively, showed no significant beta-galactosidase activity compared to those same cells that were untreated shown in lane 10. SK HEPL cells responded similarly to the receptor (-) Morris 7777 cells.

In the staining procedure described in Example 1, Hep G2 cells treated with the conjugated virus produced a bluish coloration as did the Huh-7 cells treated similarly. However, cells that did not receive treatment had no staining.

EXAMPLE 2

Chemical Modification and Alteration of Host Cell Specificity of Hepatitis B Virus (HBV)

Hepatitis B virus is a human pathogen that possesses very narrow host (species) and organ (liver) specificities. In vitro, the virus is also very fastidious as evidenced by the fact that human hepatocytes or hepatoma cells in culture cannot be infected by HBV without unusual and highly artificial conditions such as high concentrations of corticosteroids.

Cells and Cell Culture

Hepatitis B virus (HBV) was obtained from Hep G2 producer cells chronically infected with HBV as described by Sells et al. Proc. Natl. Acad. Sci. 84:1005-1009 (1987), and maintained in Dulbecco's modified Eagle's medium (MEM) containing G418 as 380 mg/ml, supplemented with 10% heat inactivated fetal bovine serum. To test the infectivity and specificity of unmodified and modified HBV, two human cell lines were cultured. Huh7 human hepatoma cell line which possesses asialoglycoprotein receptors and IMR-90 fibroblasts which do not possess asialoglycoprotein receptors were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS).

Isolation of HBV

HepG2 cells were cultured in serum free media for three days. The medium was centrifuged at 2000 rpm to remove debris and the supernatant applied on 10-20% lactose gradient, pH 7.4, 8.0 or 8.4, and ultracentrifuged at 40000 rpm in VTi55 rotor at 4°C for 16 hours to pellet and isolate the virus.

Chemical Modification of HBV

HBV obtained (3.0 mg of protein) was lactosaminated in a similar fashion to that described in Example 1 using 10 mg of sodium cyanoborohydride for 3 hours at 25°C. The modified virus was sterilized by filtration through 0.45 µm membranes and then dialyzed against MEM through membranes with a 12-14000 molecular weight exclusion limit followed by dialysis against MEM plus 10% FBS.

Infection of Cells with Unmodified and Modified HBV

Huh7 and IMR-90 cells were plated at 25-50% confluence in 35 or 100 mm diameter plastic dishes. Cell medium was removed and replaced with medium containing modified or unmodified virus and incubated at 37°C. Cells were washed and changed to fresh medium every three days and at regular intervals cells were studied for the presence of HBV DNA and medium analyzed for the presence of hepatitis B surface antigen (HBsAg).

Detection of Targeted HBV DNA in Huh7 Cells Treated with Modified and Unmodified HBV

DNA was extracted from cells according to the method by Blin, N. and Stafford, D.W. Nucleic Acid Res. 3:2303-2312 (1976), in which the cells were washed twice with 10 ml of cold Tris-buffered saline (TBS), scraped off into TBS and centrifuged at 200 rpm. The cell pellet was resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, was added to the same buffer containing 20 mg/ml RNase, 0.5% SDS, and then treated with proteinase K. Cellular DNA was isolated by ethanol precipitation after phenol extraction. The DNA was analyzed by Southern blot using a γ^{32}P-ATP labeled cDNA probe specific for HBV sequences (a Bam HI restriction fragment of plasmid adw HTD carrying the HBV genome, obtained from Dr. Jake Liang, Massachusetts General Hospital).

The Southern blot showed no hybridizable sequences when probed with our cDNA probe specific for HBV. This confirms the previous finding that Huh7 cells, even though of human origin, cannot be infected by unmodified HBV under the conditions of

routine cell culture. In addition, the data indicate that the washing procedures eliminate any detectable non-specifically bound HBV DNA on these cells. However, treatment of the Huh7 cells with modified HBV for as little as one day of incubation resulted in a strong signal of hybridizable bands on the Southern blot corresponding to those expected for the plasmid sequences. IMR-90, asialoglycoprotein (-) cells did not produce hybridizable sequences under any conditions.

Detection of HBsAg in the Supernatant of Huh7 and IMR-90 Cells Exposed to Unmodified or Modified HBV

Medium from Huh7 and IMR-90 cells was incubated with modified or unmodified HBV as described above and at various intervals was assayed for HBsAg by an enzyme immunoassay kit (Auszyme Monoclonal). The conditions were those recommended by the manufacturer, Abbott Labs.

As shown in Table 4, the background color absorbance was approximately 0.121 in untreated Huh7 cells and there was no significant difference between day 1 and day 7. Unmodified HBV did not result in significant production of HBsAg. Absorbance here was approximately 0.180. Similarly, the color absorbance reflecting HBV levels in IMR-90 cells did not exceed 0.110. However, Huh7 cells treated with modified HBV released HBsAg into their supernatants, with absorbance ranging from 0.760 to 0.865.

Table 4

Levels of Hepatitis B Surface Antigen (HBsAg) in Culture Medium as Determined by Auszyme Assay (Absorbance Units)

	Cells			
	IMR-90		Huh7	
Day	Modified HBV	Untreated	Unmodified HBV	Modified HBV
1		.121 ± .054	.135 ± .017	.850 ± .010
3			.186 ± .036	.700 ± .012
5			.171 ± .010	.865 ± .053
7	.110 ± .023			.764 ± .067

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

CLAIMS

1. A method of targeting a virus or a cell for internalization into a target cell, comprising introducing onto the surface of the virus, or cell, a molecule which binds to a surface receptor of the target cell to produce a modified virus or cell which binds to the receptor, is internalized selectively by the cell *in vivo* and expresses the delivered nucleic acid.

2. The method of claim 1, wherein the virus or cell is a bacterium, a protozoan or a mammalian cell.

3. The method of claim 1, wherein the virus or cell, in unmodified form, is not normally internalized by the target cell.

4. The method of claim 3, wherein the virus or cell is a human pathogen and the target cell is a nonhuman cell.

5. A method of targeting the internalization of a virus or viral component into a target cell, comprising introducing onto the surface of the virus or viral component a molecule which binds to a receptor of the target cell to produce a modified virus or viral component which binds to the receptor and is internalized selectively by the cell.

6. The method of claim 5, wherein the virus is infective.

7. The method of claim 5, wherein the virus or viral component is replication defective.

8. The method of claim 5, wherein the virus is a retrovirus.

9. The method of claim 5, wherein the virus, or viral component, in unmodified form, does not infect the cell.

10. The method of claim 9, wherein the virus is a human pathogen and the target cell is a nonhuman cell.

11. The method of claim 5, wherein the virus is a pathogen for hepatocytes.

12. The method of claim 10, wherein the virus is a hepatitis virus.

13. The method of claim 12, wherein the receptor mediates endocytosis of the molecule by the cell.

14. The method of claim 13, wherein the receptor is an asialoglycoprotein receptor, the molecule introduced onto the surface of the virus, or viral component, is a ligand for the asialoglycoprotein receptor and the targeted cell bears an asialoglycoprotein receptor.

15. The method of claim 13, wherein the ligand for the asialoglycoprotein receptor is galactose or N-acetyl galactosamine and the target cell bearing an asialoglycoprotein receptor is an hepatocyte.

16. The method of claim 5, wherein the molecule is introduced onto the surface of the virus or viral component by chemical coupling.

17. A method of targeting the infectivity of a virus to a cell bearing an asialoglycoprotein receptor, comprising introducing onto the surface of the virus a ligand for the asialoglycoprotein receptor to produce a modified virus which infects a cell bearing asialoglycoprotein receptor.

18. The method of claim 17, wherein the ligand for the asialoglycoprotein receptor is lactose or galactose.

19. The method of claim 17, wherein the cell bearing the asialoglycoprotein receptor is an hepatocyte.

20. The method of claim 17, wherein the virus is a human pathogen and the cell is a non-human cell.

21. The method of claim 20, wherein the virus is hepatitis virus.

22. A modified virus, or component thereof, having on its surface a molecule which binds to a surface component of a cell which is not normally infectable by the virus in its unmodified form, the modified virus, or component thereof, being capable of binding to and being internalized by the cell.

23. The modified virus of claim 22, wherein the cellular surface component of the cell is a receptor which mediates endocytosis by the cell.

24. The modified virus of claim 23, wherein the receptor is an asialoglycoprotein receptor and the molecule is a ligand for the asialoglycoprotein receptor.

25. The modified virus of claim 22, which is a human pathogen.

26. The modified virus of claim 25, which is a hepatitis virus.

27. Modified hepatitis virus containing lactose or galactose terminal carbohydrates on its surface.

28. A method of introducing nucleic acid into a cell, comprising:

a) incorporating the nucleic acid into a viral vector comprising a modified virus, or viral component, containing a molecule on its surface which binds to a surface component of the cell; and

b) contacting the viral vector and the cell under conditions which allow the vector to become internalized by the cell and expresses the introduced nucleic acid.

29. The method of claim 28, wherein the virus, or component thereof, in unmodified form, does not ordinarily infect the cell.

30. The method of claim 28, wherein the nucleic acid is an expressible gene.

31. The method of claim 28, wherein the virus is a retrovirus.

32. The method of claim 28, wherein the molecule introduced onto the surface of a virus or viral component is a galactose derivative, the cellular surface component is a ligand for the asialoglycoprotein receptor and the cell bears an asialoglycoprotein receptor.

33. The method of claim 32, wherein the cell bearing an asialoglycoprotein receptor is an hepatocyte.

34. A method of infecting an animal cell with a human virus that, in unmodified form, does not normally infect the animal cell, comprising providing a modified human virus having on its surface a molecule which binds to a surface component of the animal cell, the modified human virus being capable of binding to and infecting the animal cell and contacting the modified virus and the cell under conditions which allow the modified virus to bind to and infect the cell.

35. The method of claim 34, wherein the human virus is a human pathogen.

36. The method of claim 34, wherein the animal cell and the modified virus are contacted in vivo.

37. The method of claim 34, wherein the animal cell and the modified virus are contacted in vitro.

38. An animal cell infected with a modified human virus, the cell being uninfectable by the virus in unmodified form.

39. The animal cell of claim 38, comprising an hepatocyte infected with hepatitis virus.

40. An animal infected with a modified human virus, the animal being uninfectable by the virus in unmodified form.

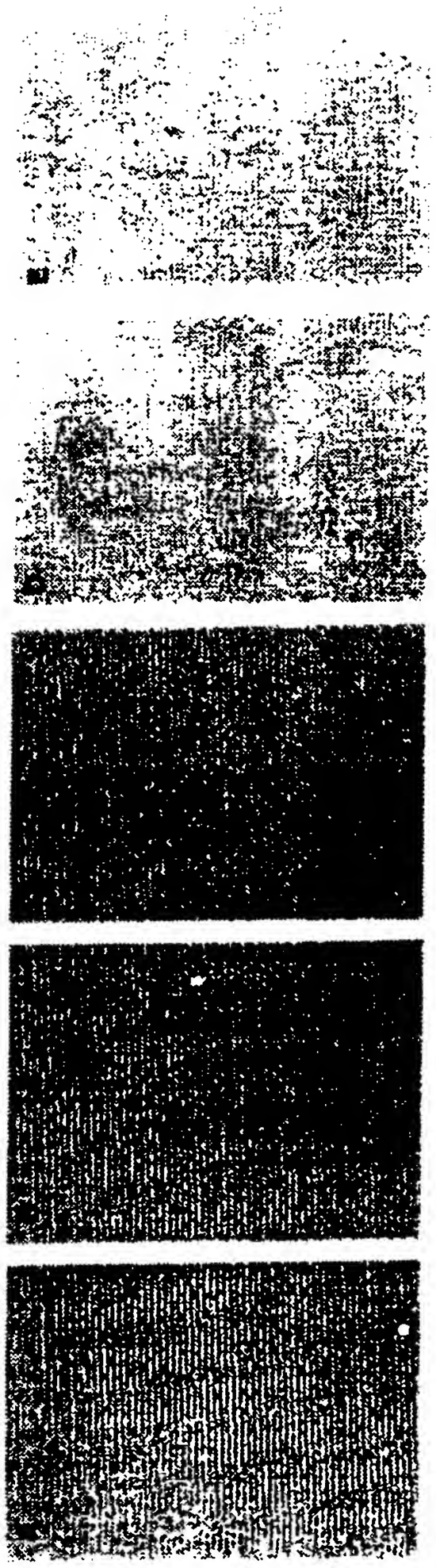


FIG. IA FIG. IB FIG. IC FIG. ID FIG. IE

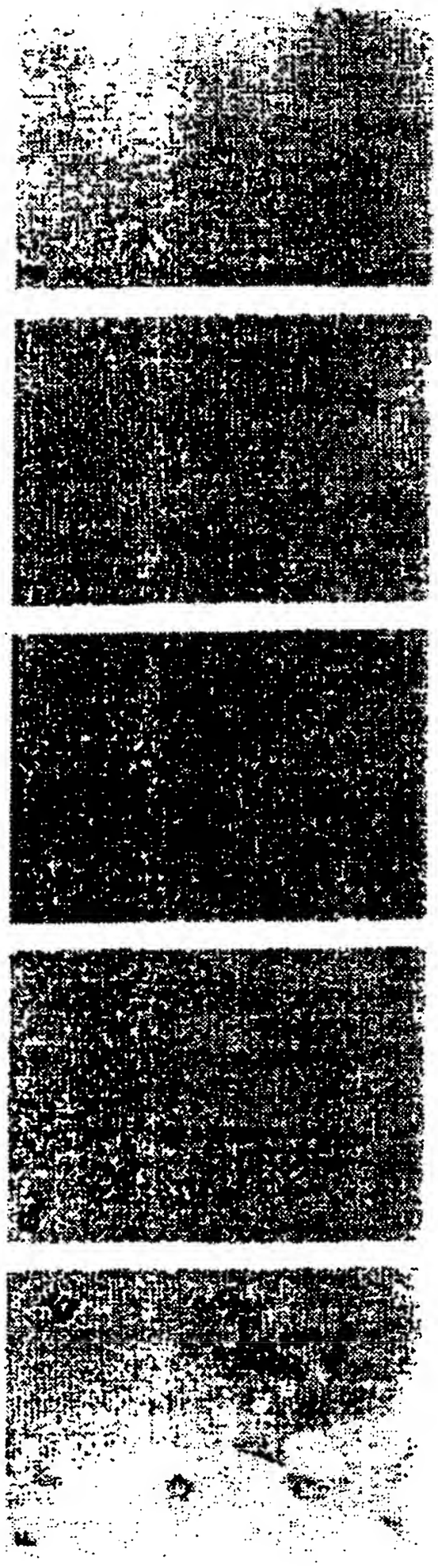


FIG. IF FIG. IG FIG. IH FIG. II FIG. IJ

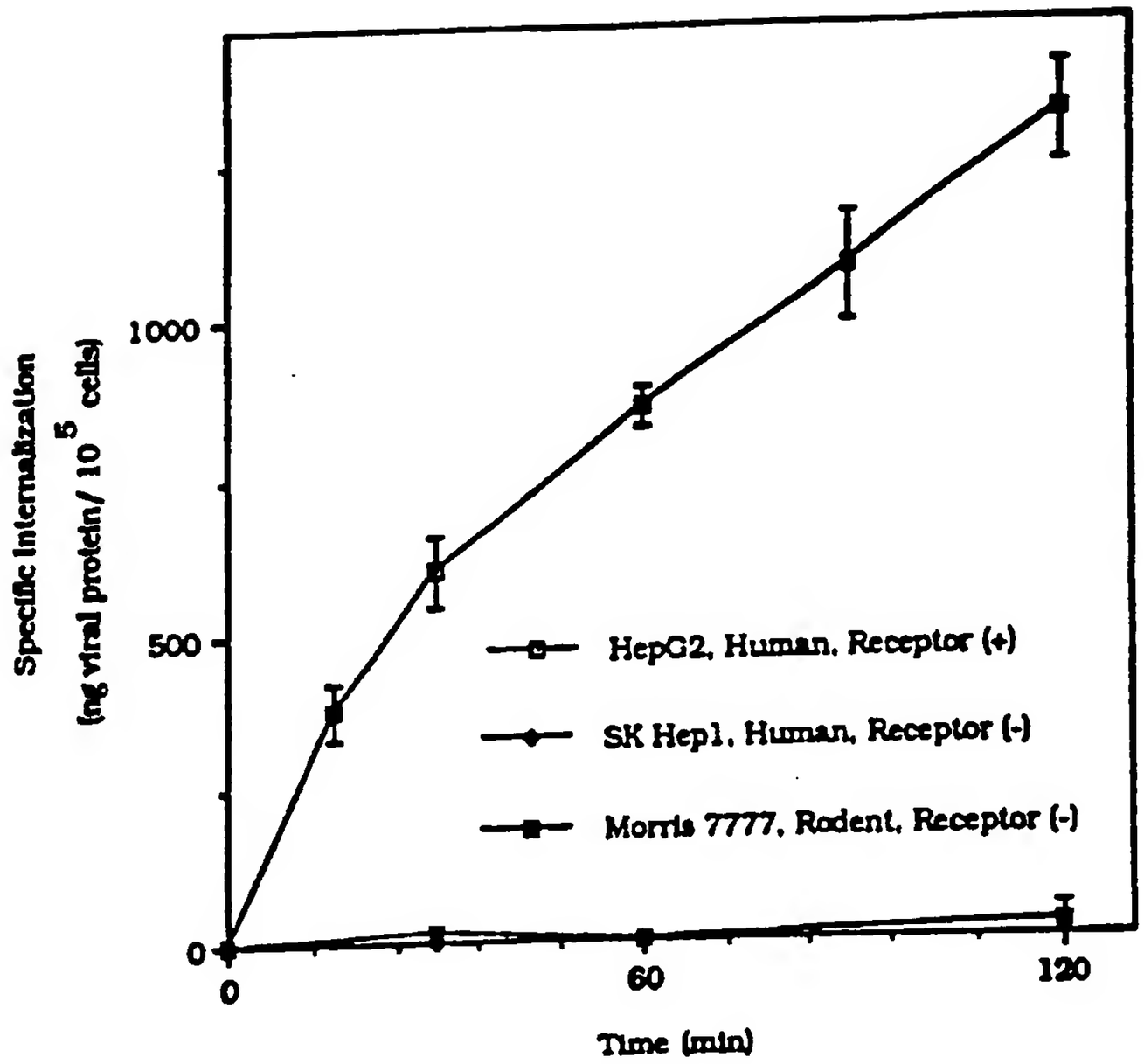

Specific Internalization
(ng viral protein/ 10^5 cells)
1000
500
0
0
60
120
Time (min)
HepG2, Human, Receptor (+)
SK Hep1, Human, Receptor (-)
Morris 7777, Rodent, Receptor (-)

Figure 2

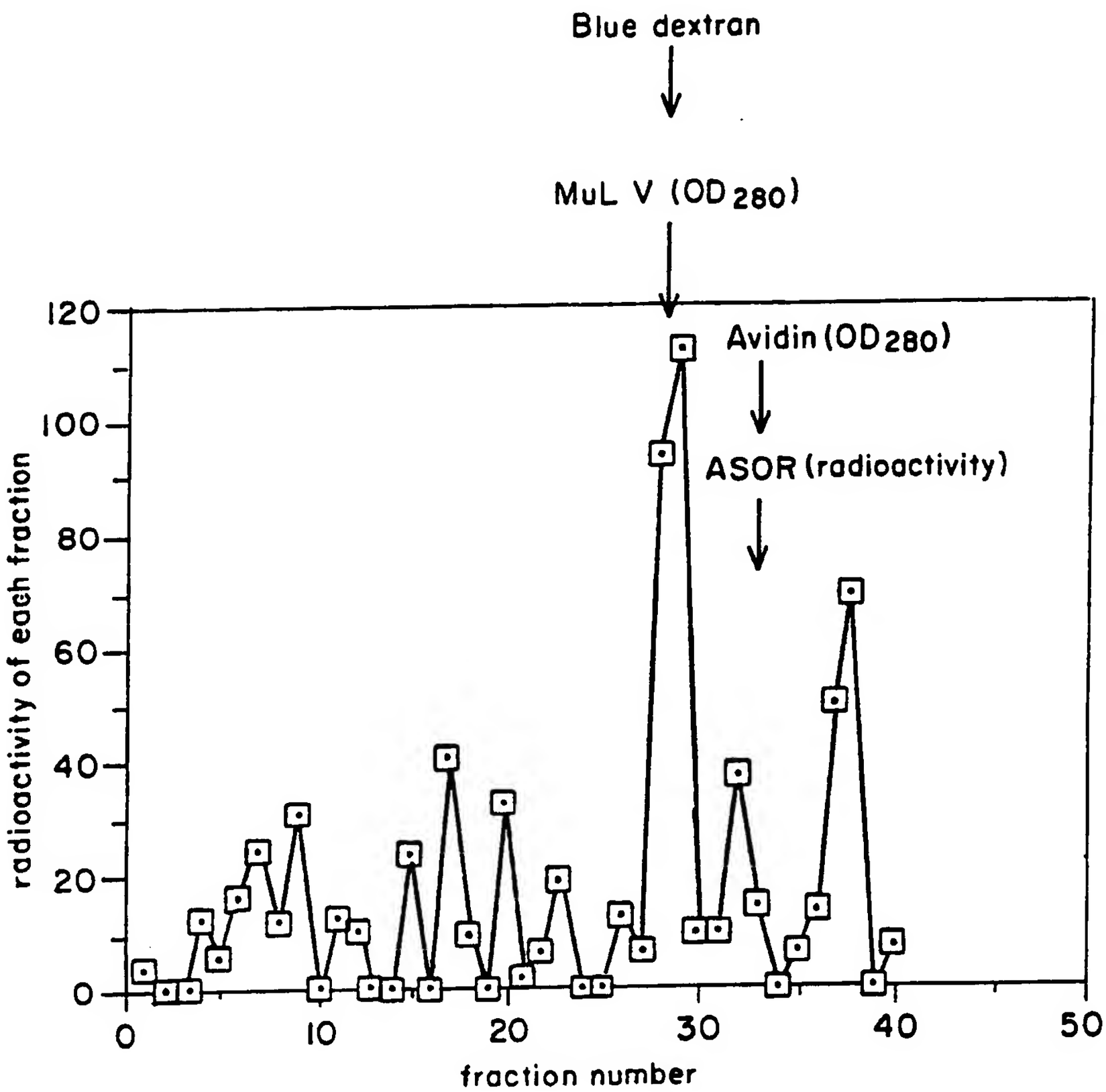
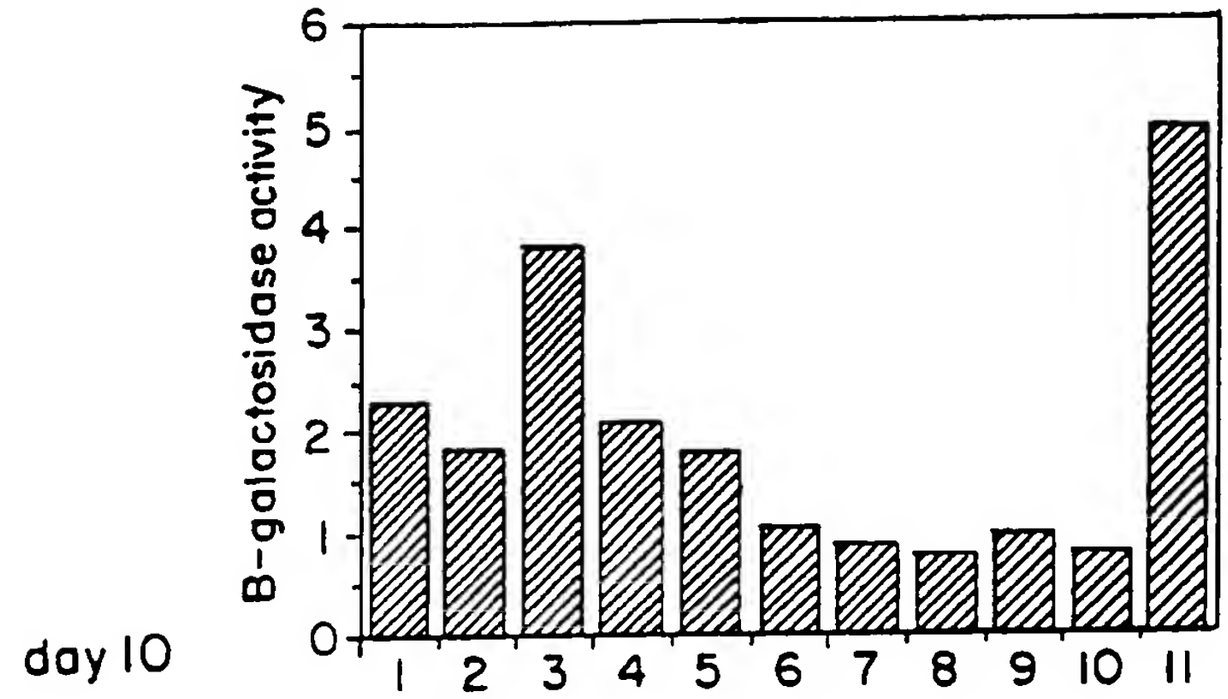

Blue dextran
MuL V (OD 280)
Avidin (OD 280)
ASOR (radioactivity)
radioactivity of each fraction
fraction number
0
20
40
60
80
100
120
0
10
20
30
40
50

FIG. 3



HepG2
1: conj. 2.29 ± 0.16
2: no treatment 1.81 ± 0.01

HUH-7
3: conj. 3.80 ± 0.11
4: bio-MLV 2.09 ± 0.05
5: no treatment 1.76 ± 0.00

Mahlavu
6: conj. 1:04 ± 0.13
7: no treatment 0.89 ± 0.09

MC 7777
8: conj. 0.75 ± 0.01
9: bio-MLV 0.99 ± 0.38
10: no treatment 0.80 ± 0.07

BAG
11: no treatment 5.00 ± 0.08

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application N. PCT/US91/07103

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 7/00, 11/02, 11/06, 15/00; A01N 63/00; A61K 37/00;
USCL: 435/172.3, 177, 181, 235, 320.1; 424/93; 800/2

II. FIELDS SEARCHED

Minimum Documentation Searched *

Classification System	Classification Symbols
U.S.CL.	435/172.3, 177, 181, 320.1, 235; 424/93; 800/2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched *

Database: Dialog (411)
Automated Patent System (File US PAT 1971-1991)

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	Proc. Natl. Acad. Sci. USA, Vol. 86, issued December 1989, Roux et al., "A versatile and potentially general approach to the targeting of specific cell types by retroviruses: Application to the infection of human cells by means of major histocompatibility complex class I and class II antigens by mouse ecotropic murine leukemia virus-derived viruses", pages 979-982, See Figs. 1-2 and pages 9080-9081.	1-3, 5-9, 22, 28-31, 34

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 December 1991	04 FEB 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Gian Wang

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Virology, Vol. 172, issued 1989, Pugh et al., "Infection and Uptake of Duck Hepatitis B Virus by Duck Hepatocytes Maintained in the Presence of Dimethyl Sulfoxide", pages 564-572, See Figs. 1-8, pages 565-571.	1-40
Y	J. Gen. Virol., Vol. 68, issued 1987, Grundy, et al., B_2 Microglobulin Enhances the Infectivity of Cytomegalovirus and when Bound to the Virus Enables Class I HLA Molecules To Be Used as a Virus Receptor", pages 793-803, See Figs. 1-8 and Tables 1-2, pages 795-799.	1-40
Y	The Journal of Biological Chemistry, Vol. 263, No. 29, issued 15 October 1988, Wu et al., "Receptor - mediated Gene Delivery and Expression in Vivo", pages 14621-14624, See the entire document.	1-40

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Virology, Vol. 163, issued 1988, Komai et al., "The Vero Cell Receptor for the Hepatitis B Virus Small S Protein is a Sialoglycoprotein", pages 629-634, See Figs. 1-2, Tables 1-3, pages 630-631 and 633.	1-40

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [4]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.